

10/019156
PATENT

Attorney Docket No.: 5885.204-US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE
(DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

Box PCT
Commissioner for Patents
Washington, DC 20231

Sir:

This is a request for filing a patent application under 35 U.S.C. 371 of the international application identified below:

INTERNATIONAL APPLICATION NO. PCT/DK00/00300	INTERNATIONAL FILING DATE June 2, 2000	PRIORITY DATE CLAIMED June 2, 1999
APPLICANT(S)/INVENTOR(S)		
First Name	Middle Name	Family Name
1.Thomas 2.Shamkant 3.Allan 4. Jesper	1.Honger 2.Anant 3. 4.	1.Callisen 2.Patkar 3.Svensen 4.Vind
TITLE OF THE INVENTION		
Chemically Modified Lipolytic enzyme		

1. This is a first submission of items concerning a filing under 35 U.S.C. 371.
2. This is an express request to begin national examination procedures (35 U.S.C. 371(f)).
3. The US has been elected by the expiration of 19 months from the priority date (Article 31).
4. A copy of the International Application as filed (35 U.S.C. 371(c)(2)) has been communicated by the International Bureau.
5. An application data sheet is enclosed.
6. Priority of Danish application no. PA 1999 00778 filed on June 2, 1999 is claimed under 35 U.S.C. 119(a)-(d).
7. The benefit of U.S. provisional application no. 60/138,081 filed on August 6, 1999 is claimed under 35 U.S.C. 119(e).
8. An oath or declaration of the inventors is submitted herewith.
9. An Information Disclosure Statement is submitted herewith.
10. An Assignment and Recordation Form Cover Sheet are submitted herewith.

11. A Preliminary Amendment is submitted herewith.
12. The assignee of the present application is:

Novozymes A/S
Krogshoejvej 36
DK-2880 Bagsvaerd
Denmark

Applicants request that the assignee information be included in the published patent application.

13. Direct all correspondence to Customer Number 25908:

PATENT TRADEMARK OFFICE



25908

14. The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)):

Search Report has been prepared by the EPO \$890.00

CLAIMS FEE

Total Claims: $17 - 20 = 0 \times 18 =$ \$0.00

Independent Claims: $6 - 3 = 3 \times 84 =$ \$252.00

Total Claims Fee: \$0.00

FEE FOR RECORDING ASSIGNMENT \$40.00

TOTAL NATIONAL FEE \$1,182.00

15. Please charge the required fee, estimated to be \$1,182, to Novozymes North America, Inc., Deposit Account No. 50-1701. A duplicate of this sheet is enclosed.

Respectfully submitted,

Date: December 3, 2001

Jason I. Garbell, Reg. No. 44,116
Novozymes North America, Inc.
405 Lexington Avenue, Suite 6400
New York, NY 10174-6401
(212) 867-0123

10/019156

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03 DEC 2001

Attorney Docket No.: 5885.204-US

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Callisen et al.

Confirmation No: To be assigned

Serial No.: To be assigned

Group Art Unit: To be assigned

Filed: December 3, 2001

Examiner: To be assigned

For: Chemically Modified Lipolytic enzyme

PRELIMINARY AMENDMENT

Commissioner for Patents
Washington, DC 20231

Sir:

Before examination, please amend the above-identified application as follows (a marked up version pursuant to 37 C.F.R. 1.21 is attached hereto):

IN THE SPECIFICATION:

At page 1, after the title, insert

--CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a 35 U.S.C. 371 national application of PCT/DK00/00300 filed June 2, 2000 (the international application was published under PCT Article 21(2) in English) and claims, under 35 U.S.C. 119, priority of Danish application no. PA 1999 00778 filed June 2, 1999 and the benefit of U.S. provisional application no. 60/138,081 filed August 6, 1999, the contents of which are fully incorporated herein by reference.--

IN THE CLAIMS:

Please substitute the following amended claims for the pending claims having the same claim numbers:

Claims

3. (Amended) The lipolytic enzyme of claim 1 wherein the parent lipolytic enzyme has an amino acid sequence having one, two or three amino groups, and wherein the hydrophobic group(s) is/are covalently linked to the amino group(s).

4. (Amended) The lipolytic enzyme of claim 1 wherein the hydrophobic group is a fatty acyl group, a polyalkoxy or an alkyl-polyalkoxy group.
5. (Amended) The lipolytic enzyme of claim 1 wherein the parent lipolytic enzyme belongs to the *Humicola* group.
6. (Amended) The lipolytic enzyme of claim 1 wherein the lipolytic enzyme is a lipase, a cutinase or a phospholipase.
9. (Amended) The method of claim 10 wherein the modification results in one, two or three amino, thiol, hydroxy or carboxy groups.
10. (Amended) The method of claim 8 wherein the modification comprises modification of the amino acid sequence by site-directed mutagenesis.
11. (Amended) The method of claim 8 wherein the modification comprises substitution of a lysine residue with another amino acid and/or substitution of another amino acid residue with lysine and/or chemical modification to remove N-terminal amino group, and the hydrophobic group is linked to amino groups.
12. (Amended) The method of claim 11 wherein the modification results in 1-3 amino groups in the enzyme molecule.
14. (Amended) The detergent composition of claim 13 wherein the hydrophobic group is fatty acyl group.
15. (Amended) The detergent composition of claim 13 wherein the lipolytic enzyme has one, two or three hydrophobic groups covalently linked.

REMARKS

This amendment is submitted to cancel claims in order to reduce the filing fee. There is no new matter added, and entry of the amendment is respectfully requested.

The Examiner is hereby invited to contact the undersigned by telephone if there are any questions concerning this amendment or application.

Respectfully submitted,

Date: December 3, 2001



Jason I. Garbell, Reg. No. 44,116
Novozymes North America, Inc.
405 Lexington Avenue, Suite 6400
New York, NY 10174-6401
(212) 867-0123

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For: Chemically Modified Lipolytic enzyme

VERSION WITH MARKINGS TO SHOW CHANGES MADE

Sir:

Below is a marked-up version of the amendments made in the accompanying amendment.

IN THE CLAIMS:

Claims 3-6, 9-12, and 14-15 have been amended as follows:

3. (Amended) The lipolytic enzyme of claim 1 [or 2] wherein the parent lipolytic enzyme has an amino acid sequence having one, two or three amino groups, and wherein the hydrophobic group(s) is/are covalently linked to the amino group(s).
4. (Amended) The lipolytic enzyme of [any preceding claim] claim 1 wherein the hydrophobic group is a fatty acyl group, a polyalkoxy or an alkyl-polyalkoxy group.
5. (Amended) The lipolytic enzyme of [any preceding claim] claim 1 wherein the parent lipolytic enzyme belongs to the *Humicola* group[, particularly *Humicola lanuginosa* lipase].
6. (Amended) The lipolytic enzyme of [any preceding claim] claim 1 wherein the lipolytic enzyme is a lipase, a cutinase or a phospholipase.
9. (Amended) The method of [the preceding claim] claim 10 wherein the modification results in one, two or three amino, thiol, hydroxy or carboxy groups[, particularly located in the lipid contact zone of the parent lipolytic enzyme].
10. (Amended) The method of claim 8 [or 9] wherein the modification comprises modification of the amino acid sequence by site-directed mutagenesis.

11. (Amended) The method of [any of claims 8-10] claim 8 wherein the modification comprises substitution of a lysine residue with another amino acid [(particularly arginine or histidine)] and/or substitution of another amino acid residue with lysine and/or chemical modification to remove N-terminal amino group, and the hydrophobic group is linked to amino groups.
12. (Amended) The method of [the preceding claim] claim 11 wherein the modification results in 1-3 amino groups in the enzyme molecule.
14. (Amended) The detergent composition of [the preceding claim] claim 13 wherein the hydrophobic group is fatty acyl group.
15. (Amended) The detergent composition of claim 13 [or 14] wherein the lipolytic enzyme has one, two or three hydrophobic groups covalently linked.

CHEMICALLY MODIFIED LIPOLYTIC ENZYME**FIELD OF THE INVENTION**

The present invention relates to a chemically modified lipolytic enzyme, its preparation and its uses thereof.

5 BACKGROUND OF THE INVENTION

Lipolytic enzymes such as lipases and phospholipases are used, e.g., in detergents and baking.

Thus, lipases have been used for a number of years as detergent enzymes to remove lipid or fatty stains from clothes and other textiles, particularly a lipase derived from *Humicola lanuginosa* (EP 258 068 and EP 305 216) sold under the trade
10 name Lipolase® (product of Novo Nordisk A/S).

Fatty acid-modified lipases and their use in transesterification have been described. M. Murakami et al., JAOCS, 70 (6), 571-574 (1993); K. Green et al., JAOCS, 75 (11), 1519-1526 (1998).

15 It is also known to add lipases and phospholipases to breadmaking dough. WO 94/04035; WO 98/26057.

SUMMARY OF THE INVENTION

The inventors have developed lipolytic enzymes which are chemically modified by covalently linking one or more hydrophobic groups to the enzyme molecule.
20 They found that the chemical modification may improve the performance of the lipolytic enzyme, e.g., in baking or in detergents. The benefits may include improved thermostability and an altered substrate specificity. A modified lipase or cutinase may show improved detergency, particularly improved first-wash performance, whiteness maintenance, dingy cleanup, and reduced formation of fatty acids during
25 the drying process with less risk of forming an unpleasant smell. The benefits in baking include an increased loaf volume.

Accordingly, the invention provides a lipolytic enzyme which is chemically modified by having one or more (particularly 1-3) hydrophobic groups covalently

linked to the enzyme. The invention also provides us of such modified lipolytic enzyme in detergents and baking.

The invention further provides a method of preparing a chemically modified lipolytic enzyme by covalently linking hydrophobic groups to a parent lipolytic enzyme. Optionally, the amino acid sequence of the enzyme may be modified before the covalent linking.

DETAILED DESCRIPTION OF THE INVENTION

Parent lipolytic Enzyme

The lipolytic enzyme is an enzyme classified under the Enzyme Classification number E.C. 3.1.1.- (Carboxylic Ester Hydrolases) in accordance with the Recommendations (1992) of the International Union of Biochemistry and Molecular Biology (IUBMB). Thus, the lipolytic enzyme may exhibit hydrolytic activity, typically at a water/lipid interface, towards carboxylic ester bonds in substrates such as mono-, di- and triglycerides, phospholipids, thioesters, cholesterol esters, wax-esters, cutin, suberin, synthetic esters or other lipids mentioned in the context of E.C. 3.1.1. The lipolytic enzyme may, e.g., have activity lipase activity (with triglycerides as substrate), phospholipase activity, esterase activity or cutinase activity.

The parent lipolytic enzyme may be prokaryotic, particularly a bacterial enzyme, e.g. from *Pseudomonas*. Examples are *Pseudomonas* lipases, e.g. from *P. cepacia*, *P. glumae*, *P. pseudoalcaligenes* and *Pseudomonas* sp. strain SD 705. Other examples are bacterial cutinases, e.g. from *Pseudomonas* such as *P. mendocina* (US 5,389,536) or *P. putida* (WO 88/09367).

Alternatively, the parent lipolytic enzyme may be eukaryotic, e.g. fungal, such as lipolytic enzymes of the *Humicola* family and the *Zygomycetes* family and fungal cutinases. Examples of fungal cutinases are the cutinases of *Fusarium solani pisi* and *Humicola insolens*.

The *Humicola* family of lipolytic enzymes consists of the lipase from *H. lanuginosa* strain DSM 4109 and lipases having more than 50 % homology with said lipase. The lipase from *H. lanuginosa* (synonym *Thermomyces lanuginosus*) is de-

scribed in EP 258 068 and EP 305 216, and has the amino acid sequence shown in positions 1-269 of SEQ ID NO: 2 of US 5,869,438.

The *Humicola* family also includes the following lipolytic enzymes: lipase from *Penicillium camembertii*, lipase/phospholipase from *Fusarium oxysporum*, li-
5 pase from *F. heterosporum*, lysophospholipase from *Aspergillus foetidus*, phospholi-
pase A1 from *A. oryzae*, lipase from *A. oryzae*, lipase/ferulic acid esterase from *A. niger*, lipase/ferulic acid esterase from *A. tubingensis*, lipase from *A. tubingensis*,
lysophospholipase from *A. niger* and lipase from *F. solani*.

The *Zygomycetes* family comprises lipases having at least 50 % homology
10 with the lipase of *Rhizomucor miehei*. This family also includes the lipases from *Ab-
sidia reflexa*, *A. sporophora*, *A. corymbifera*, *A. blakesleeana*, *A. griseola* and
Rhizopus oryzae.

The phospholipase may have A₁ or A₂ activity to remove fatty acid from the
phospholipid and form a lyso-phospholipid, or it may be have phospholipase B or
15 lysophospholipase activity. It may or may not have lipase activity, i.e. activity on
triglycerides. The phospholipase may be of animal origin, e.g. from pancreas (e.g.
bovine or porcine pancreas), snake venom or bee venom. Alternatively, the phos-
pholipase may be of microbial origin, e.g. from filamentous fungi, yeast or bacteria,
such as the genus *Aspergillus*, *Fusarium* or *Hyphozyma* (WO 98/18912), particularly
20 the species *A. niger* or *F. oxysporum* (WO 98/26057).

Other examples of lipolytic enzymes are described in PCT/DK 99/00664
(Danish patent application PA 1998 01572).

The lipolytic enzyme may be native to such source, or it may be a variant
thereof obtained by altering the amino acid sequence. Examples of such variants are
25 those described in WO 92/05249, WO 94/25577, WO 95/22615, WO 97/04079 and
WO 97/07202, WO 98/08939, PCT/DK 99/00068, EP 99610010.3 and Danish patent
application PCT/DK 00/00156 (PA 1999 00441). A specific example is a variant of the
Humicola lanuginosa lipase having the mutations E1SPPCGRRP +E99N +N101S
+E239C +Q249R.

Hydrophobic group

Generally, a hydrophobic group can be identified from a negative free-energy-of-transfer from water to oil. More specifically, suitable hydrophobic groups can be identified in a partition coefficient experiment where the two media are an aqueous detergent solution and a surface containing the (lipid) substrate of choice. The general concept is described in standard text books such as C. Tanford (1980), The hydrophobic effect, Wiley, New York.

The hydrophobic group may be a fatty acyl group, particularly having 12-22 or 14-20 carbon atoms, straight-chain or branched, saturated, mono- or polyunsaturated, optionally substituted. Examples are myristoyl (tetradecanoyl), palmitoyl (hexadecanoyl), stearoyl (octadecanoyl) and arachidoyl (eicosanoyl).

Other examples of hydrophobic groups are those commonly found in surfactants, e.g. a hydrophobic polymer group such as poly-alkoxy or alkyl-polyalkoxy of the general formula $R^1-(O-CHR^2-CH_2)_n$ wherein R^1 is H or $C_{14}-C_{22}$ alkyl, R^2 is H or methyl, and n is 10-200, e.g. 20-100.

The hydrophobic group(s) may particularly be linked to an amino acid in the lipid contact zone of the lipolytic enzyme (as described in WO 92/05249) or within 5 Å from the edge of said zone.

The modified lipolytic enzyme containing one, two or three hydrophobic groups will be referred to as a monopod, dipod or tripod, respectively.

Covalent linking

The hydrophobic group may be covalently linked, e.g., to an amino group (lysine or N-terminal), a thiol group (cysteine residues), a hydroxyl group (serine or threonine) or a carboxyl group (glutamic acid, aspartic acid or C-terminal) in the amino acid sequence of the lipolytic enzyme. The covalent linking can be done by methods known in the art.

Thus, linking to amino groups can be done through a reactive intermediate such as an N-hydroxy-succinimide activated fatty acids, e.g. stearoyl or arachidoyl acid N-hydroxy-succinimide, or maleimide esters at high pH (e.g. pH 8-9).

Linking to a thiol group can be done by linking to a maleimide ester at pH 6.5-7, by reaction with fatty acid methane thiosulfonate (e.g. at pH 8), or as described in WO 91/16423, WO 98/23732 or WO 99/37323.

Linking to a carboxyl group can be done by linking a hydrophobic amine as
5 described in WO 95/09909.

To ensure that the number of hydrophobic groups linked to each enzyme molecule will be from one to three, one strategy uses a lipolytic enzyme having an amino acid sequence with one, two or three of the group in question (e.g. amino or thiol). This is discussed below.

10 Another strategy is to choose the conditions (amounts of reagents etc.) for the linking reaction such that, on average, 1-3 hydrophobic groups will be linked to each enzyme molecule.

Amino acid sequence

A lipolytic enzyme with 1-3 groups may be a variant obtained by modifying
15 the amino acid sequence of a given lipolytic enzyme by recombinant technology using site-directed mutagenesis.

Thiol groups can also be introduced by chemical reaction as described in Duncan et al., (1983) Anal. Biochem. 132, 68-73.

The N-terminal amino group may be eliminated by using site-directed
20 mutagenesis to change the N-terminal to glutamine and after expression convert this to pyroglutamate by cyclization (Thiede, B., Lamer S., Mattow J., Siejak F., Dimmler C., Rudel T., Jungblut PR.; rapid communications in Mass spectroscopy Vol 14 (6) pp.496-502 (2000). A choice for expression of pyroglutamate containing peptide in filamentous fungi, could be to use parts of the signal peptide and N-terminal of the
25 peroxidase from the filamentous fungi *Coprinus cinereus*. This peroxidase has an N-terminal pyroglutamate (Baunsgaard L., Dalboge H., Houen G., Rasmussen EM, Welinder KG.; European journal of Biochemistry vol. 213 (1) 605-611 (1993).

The peroxidase N-terminal and part of the neighboring amino acids can be conferred to the N-terminal of the lipolytic enzyme by standard molecular biological
30 techniques to create a variant with a pyro-glutamic N-terminal.

Lipolytic enzyme variant

The lipolytic enzyme variant may be designed to change the number and location of amino or thiol groups by amino acid insertion, deletion and/or substitution involving lysine or cysteine.

- 5 A change in the number of lysine residues may be balanced by a change in the number of other charged amino acids may, to keep the isoelectric point fairly unchanged. Thus, lysine may be substituted with another positively charged amino acid (histidine or arginine).

One strategy is to remove some of the lysine residues by substitution or deletion and keep 1-3 lysine residues unchanged. Thus, of the 6 lysine residues in the *Humicola lanuginosa* lipase, one or more of the following may be retained: K24, K98, K233.

Another strategy is to remove all lysine residues in the native lipolytic enzyme by substitution or deletion (and optionally remove the N-terminal amino group) and to introduce one, two or three lysine residues by substitution or insertion at selected positions in the lipid contact zone.

Thus, for a lipolytic enzyme of the *Humicola* family, existing amino groups may be removed, and 1-3 lysine residues may be introduced at positions corresponding to the following amino acids in the *Humicola lanuginosa* lipase: 14, 15, 17-28, 35-42, 45, 54-65, 80-85, 87-95, 110-116, 119, 144-151, 171-177, 195-209, 213-215, 219, 221-231, 234, 238, 242-251, 257-269, particularly at position 199, 56, 27, 111, 118, 37, 227, 226, 210, 95, 93, 255, 96, 252, 57 or 211.

Similarly, for a fungal cutinase, existing amino groups may be removed and 1-3 lysine residues may be introduced, e.g. by substitutions corresponding to I5K, V158K, D63K, N44K and/or R149K. Examples are I5K +V158K +D63K and N44K +V158K +D63K.

Use of modified lipolytic enzyme

The modified lipolytic enzyme can be used in any known application for such enzymes, e.g. in baking, in detergents or in immobilized form for various processes.

Baking

The modified lipolytic enzyme can be used in the preparation of dough, bread and cakes, e.g. to increase dough stability and dough handling properties, to increase the loaf volume or to improve the elasticity of the bread or cake. Thus, the enzyme can be used in a process for making bread, comprising adding the enzyme to the ingredients of a dough, kneading the dough and baking the dough to make the bread. This can be done in analogy with US 4,567,046 (Kyowa Hakko), JP-A 60-78529 (QP Corp.), JP-A 62-111629 (QP Corp.), JP-A 63-258528 (QP Corp.), EP 426211 (Unilever) or WO 99/53769 (Novo Nordisk).

10 Detergent

The lipolytic enzyme (e.g. a lipase) may be used as an additive in a detergent composition. This additive is conveniently formulated as a non-dusting granulate, a stabilized liquid, a slurry or a protected enzyme. The additive may be prepared by methods known in the art.

15 Lipases tend to exert the best fat removing effect after more than one wash cycle (Gormsen et al., in Proceedings of the 3rd World Conference on Detergents, AOCS press, 1993, pp 198-203).

Immobilized enzyme

The lipolytic enzyme may be immobilized by methods known in the art, e.g. by adsorption onto a polymer based carrier, by covalent binding to an activated polymer-based carrier (e.g. epoxy or aldehyde) and by granulation, e.g. as described in WO 89/02916, WO 90/15868, WO 95/22606 or WO 99/33964.

The immobilized lipolytic enzyme may be used for interesterification, e.g. of a water-insoluble carboxylic acid ester (such as a triglyceride) with another ester, with a free fatty acid or with an alcohol. The immobilized enzyme can also be used in ester synthesis or in resolution of racemic compounds.

DETERGENT COMPOSITION

The detergent compositions of the invention may for example, be formulated as hand and machine laundry detergent compositions including laundry additive compositions and compositions suitable for use in the pretreatment of stained fab-

rics, rinse added fabric softener compositions, and compositions for use in general household hard surface cleaning operations and dishwashing operations.

The detergent composition of the invention comprises the lipase of the invention and a surfactant. Additionally, it may optionally comprise a builder, another
5 enzyme, a suds suppresser, a softening agent, a dye-transfer inhibiting agent and other components conventionally used in detergents such as soil-suspending agents, soil-releasing agents, optical brighteners, abrasives, bactericides, tarnish inhibitors, coloring agents, and/or encapsulated or non-encapsulated perfumes.

The detergent composition according to the invention can be in liquid, paste,
10 gels, bars or granular forms. The pH (measured in aqueous solution at use concentration) will usually be neutral or alkaline, e.g. in the range of 7-11, particularly 9-11. Granular compositions according to the present invention can also be in "compact form", i.e. they may have a relatively higher density than conventional granular detergents, i.e. from 550 to 950 g/l.

15 The lipase of the invention, or optionally another enzyme incorporated in the detergent composition, is normally incorporated in the detergent composition at a level from 0.00001% to 2% of enzyme protein by weight of the composition, particularly at a level from 0.0001% to 1% of enzyme protein by weight of the composition, more particularly at a level from 0.001% to 0.5% of enzyme protein by weight of the
20 composition, even more particularly at a level from 0.01% to 0.2% of enzyme protein by weight of the composition.

The detergent composition of the invention may comprise the lipase in an amount corresponding to 10-50,000 LU per gram of detergent, particularly 20-5,000 LU/g, e.g. 100-1000 LU/g. The detergent may be dissolved in water to produce a
25 wash liquor containing lipolytic enzyme in an amount corresponding to 25-15,000 LU per liter of wash liquor, particularly 100 - 5000 LU/l, e.g. 300-2000 LU/l. The amount of lipase protein may be 0.001-10 mg per gram of detergent or 0.001-100 mg per liter of wash liquor.

More specifically, the lipase of the invention may be incorporated in the de-
30 tergents compositions described in WO 97/04079, WO 97/07202, WO 97/41212, PCT/DK WO 98/08939 and WO 97/43375.

Surfactant system

The surfactant system may comprise nonionic, anionic, cationic, ampholytic, and/or zwitterionic surfactants. The surfactant system may comprise a combination of anionic and nonionic surfactant with 70-100 % by weight of anionic surfactant and 0-30 % by weight of nonionic, particularly 80-100 % of anionic surfactant and 0-20 % nonionic or 40-70 % anionic and 30-60 % non-ionic surfactant.

The surfactant is typically present at a level from 0.1% to 60% by weight, e.g. 1% to 40%, particularly 10-40 %. particularly from about 3% to about 20% by weight. Some examples of surfactants are described below.

10 Anionic surfactants

Suitable anionic surfactants include alkyl sulfate, alkyl ethoxy sulfate, linear alkyl benzene sulfonate and mixtures of these.

The alkyl sulfate surfactants are water soluble salts or acids of the formula ROSO_3M wherein R particularly is a $\text{C}_{10}\text{-C}_{24}$ hydrocarbyl, particularly an alkyl or hydroxyalkyl having a $\text{C}_{10}\text{-C}_{20}$ alkyl component, more particularly a $\text{C}_{12}\text{-C}_{18}$ alkyl or hydroxyalkyl, and M is H or a cation, e.g., an alkali metal cation (e.g. sodium, potassium, lithium), or ammonium or substituted ammonium.

Alkylbenzene sulfonates are suitable, especially linear (straight-chain) alkyl benzene sulfonates (LAS) wherein the alkyl group particularly contains from 10 to 18 carbon atoms.

Suitable anionic surfactants include alkyl alkoxyated sulfates which are water soluble salts or acids of the formula $\text{RO(A)}_m\text{SO}_3\text{M}$ wherein R is an unsubstituted $\text{C}_{10}\text{-C}_{24}$ alkyl or hydroxyalkyl group having a $\text{C}_{10}\text{-C}_{24}$ alkyl component, particularly a $\text{C}_{12}\text{-C}_{20}$ alkyl or hydroxyalkyl, more particularly $\text{C}_{12}\text{-C}_{18}$ alkyl or hydroxyalkyl, A is an ethoxy or propoxy unit, m is greater than zero, typically between about 0.5 and about 6, more particularly between about 0.5 and about 3, and M is H or a cation which can be, for example, a metal cation (e.g., sodium, potassium, lithium, calcium, magnesium, etc.), ammonium or substituted-ammonium cation. Alkyl ethoxyated sulfates as well as alkyl propoxyated sulfates are contemplated herein. Specific examples of substituted ammonium cations include methyl-, dimethyl, trimethyl-ammonium cations and quaternary ammonium cations such as tetramethyl-ammonium and di-

methyl piperdinium cations and those derived from alkylamines such as ethylamine, diethylamine, triethylamine, mixtures thereof, and the like.

Other anionic surfactants include salts (including, for example, sodium, potassium, ammonium, and substituted ammonium salts such as mono- di- and triethanolamine salts) of soap, C₈-C₂₂ primary or secondary alkanesulfonates, C₈-C₂₄ olefinsulfonates, sulfonated polycarboxylic acids prepared by sulfonation of the pyrolyzed product of alkaline earth metal citrates.

Nonionic surfactant

The surfactant may comprise polyalkylene oxide (e.g. polyethylene oxide) condensates of alkyl phenols. The alkyl group may contain from about 6 to about 14 carbon atoms, in a straight chain or branched-chain. The ethylene oxide may be present in an amount equal to from about 2 to about 25 moles per mole of alkyl phenol.

The surfactant may also comprise condensation products of primary and secondary aliphatic alcohols with about 1 to about 25 moles of ethylene oxide. The alkyl chain of the aliphatic alcohol can either be straight or branched, and generally contains from about 8 to about 22 carbon atoms.

Further, the nonionic surfactant may comprise polyethylene oxide condensates of alkyl phenols, condensation products of primary and secondary aliphatic alcohols with from about 1 to about 25 moles of ethylene oxide, alkylpolysaccharides, and mixtures hereof, particularly C₈-C₁₄ alkyl phenol ethoxylates having from 3 to 15 ethoxy groups and C₈-C₁₈ alcohol ethoxylates (particularly C₁₀ avg.) having from 2 to 10 ethoxy groups, and mixtures thereof.

Examples of nonionic surfactants are alcohol ethoxylate, alcohol phenol ethoxylate, polyhydroxy fatty acid amide, alkyl polyglucoside and mixtures of these.

EXAMPLES

Example 1: Modified lipases with an average of 3 hydrophobic groups

Modified lipases were prepared by covalently linking tetradecanoyl (C₁₄) and hexadecanoyl (C₁₆) groups, respectively, to Lipolase (*Humicola lanuginosa* lipase). Each lipase molecule has 7 amino groups (N-terminal + 6 lysine resi-

dues), and it was estimated that an average of 3 fatty acyl groups were linked to each molecule.

Example 2: Modified lipases with 3 or 4 hydrophobic groups

Two variants of Lipolase were prepared by amino acid substitutions so that
5 the variants had the following amino groups. Other lysine residues were substituted with arginine:

Three amino groups N-terminal and lysine at positions 46 and 98.

Four amino groups: N-terminal and lysine at positions 24, 46 and 98.

Fatty acyl groups (myristoyl and stearoyl, respectively) were linked covalently
10 to the amino groups in each variant.

Example 3: Modified lipases with 2 hydrophobic groups

A variant of Lipolase was prepared by substituting lysine residues with arginine to obtain a lipase variant having two amino groups, at the N-terminal and Lys 24.

Four different modified lipases were produced by linking the following hydrophobic
15 groups to the amino groups in the variant:

Stearoyl

$C_{18}H_{37}-(O-CH_2-CH_2)_{100}$

$C_{18}H_{37}-(O-CH_2-CH_2)_{21}$

Arachidoyl

20 A similar modified lipase may be made by linking to palmitoyl groups.

Example 4: Construction of modified lipases

Monopods, dipods and tripods are prepared from Lipolase by removing the N-terminal amino group by pyroglutamate cyclization and making variants by amino acid substitutions having lysine at the following positions. Other lysine residues are
25 substituted with arginine:

Monopod: lysine at position 98, 211 or 223.

Dipod: lysine residues at positions 98 +233 or 96 +255.

Tripod: Lysine residues at positions 24 +98 +223 or at positions 57 +96 +252.

Hydrophobic groups (fatty acyl or polypropylene) are linked covalently to the lysine residues in each variant.

Example 5: First-Wash Performance

The two modified lipases were tested as described below, and unmodified
5 Lipolase was tested for comparison.

A number of variants according to the invention were tested in an anionic detergent. The experimental conditions were as follows:

	Equipment:	Thermostated Terg-o-tometer
	Method:	1 cycle wash followed by line drying.
10	Wash liquor:	1000 ml per beaker
	Swatches:	7 (cotton style # 400) swatches (9*9 cm) per beaker.
	Stain:	Lard coloured with Sudan red (0,75mg Sudan red/g lard).
15		250 µl of lard/Sudan red heated to 70°C is applied to the center of each swatch, followed by line-drying over-night.
	Water:	8.4° German hardness (°dH), Ca : Mg = 2:1
	Detergent:	1.8 g/l commercial detergent (Wisk)
	Lipase dosage:	as indicated below
20	Wash time:	20 min.
	Temperature:	30°C
	Rinse:	15 minutes in running tap water.
	Drying:	Overnight at room temperature (~ 20°C, 30-40 % RH).
25	Evaluation:	The reflectance was measured at 460 nm in a reflectometer. The results are given as ΔR (delta Reflectance) = reflectance of swatches washed in detergent with lipase minus reflectance of swatches washed in detergent without lipase.

Results:

	Lipase	Dosage, LU/l	ΔR
Reference	Lipolase	1329	0.3
		4011	0.7
Invention	Lipolase modified with C ₁₄	1617	1.9
		4880	5.2
	Lipolase modified with C ₁₆	1212	2.2
		3658	5.5

The results clearly demonstrate that the modified lipases have an improved first-wash performance.

5 Example 6: Baking tests

A chemically modified lipase was prepared by linking palmitoyl groups to Humicola lanuginosa lipase. The amounts of reagents were chosen so as to link an average of 2-3 acyl groups to each lipase molecule.

The chemically modified lipase was compared the unmodified lipase in a traditional European straight dough baking procedure.

The volume and the shape of the rolls were evaluated. Volume was evaluated by simple displacement of 10 rolls, and the shape was evaluated by measuring height/width. The results were as follows

	Invention (modified lipase)	Reference (unmodified lipase)
Lipase dosage, LU/kg flour	500	1000
Volume, ml/g	6.4	6.2
Shape (Height/width)	0.68	0.64

The results clearly show that the modified lipase at half the dosage of the reference has improved performance in terms of volume and shape.

CLAIMS

1. A lipolytic enzyme which is chemically modified by having one, two or three hydrophobic groups covalently linked to a parent lipolytic enzyme.
2. The lipolytic enzyme of claim 1 which has a hydrophobic group covalently
5 linked to an amino acid located in the lipid contact zone of the parent lipolytic enzyme or within 5 Å from the edge of said zone.
3. The lipolytic enzyme of claim 1 or 2 wherein the parent lipolytic enzyme has an amino acid sequence having one, two or three amino groups, and wherein the hydrophobic group(s) is/are covalently linked to the amino group(s).
- 10 4. The lipolytic enzyme of any preceding claim wherein the hydrophobic group is a fatty acyl group, a polyalkoxy or an alkyl-polyalkoxy group.
5. The lipolytic enzyme of any preceding claim wherein the parent lipolytic enzyme belongs to the *Humicola* group, particularly *Humicola lanuginosa* lipase.
6. The lipolytic enzyme of any preceding claim wherein the lipolytic enzyme is a
15 lipase, a cutinase or a phospholipase.
7. A method of preparing a chemically modified lipolytic enzyme, comprising covalently linking hydrophobic groups to a parent lipolytic enzyme so as to link an average of 1-3 hydrophobic groups to each enzyme molecule.
8. A method of preparing a chemically modified lipolytic enzyme, comprising:
20 a) modifying a parent lipolytic enzyme so as to change the number and/or positions of amino, thiol, hydroxy or carboxy groups, and
b) covalently linking hydrophobic groups to the amino, thiol, hydroxy or carboxy groups.

9. The method of the preceding claim wherein the modification results in one, two or three amino, thiol, hydroxy or carboxy groups, particularly located in the lipid contact zone of the parent lipolytic enzyme.

10. The method of claim 8 or 9 wherein the modification comprises modification of
5 the amino acid sequence by site-directed mutagenesis.

11. The method of any of claims 8-10 wherein the modification comprises substitution of a lysine residue with another amino acid (particularly arginine or histidine) and/or substitution of another amino acid residue with lysine and/or chemical modification to remove the N-terminal amino group, and the hydrophobic group is linked to
10 amino groups.

12. The method of the preceding claim wherein the modification results in 1-3 amino groups in the enzyme molecule.

13. A detergent composition comprising a surfactant and a lipolytic enzyme which has at least one hydrophobic group covalently linked.

15 14. The detergent composition of the preceding claim wherein the hydrophobic group is a fatty acyl group.

15. The detergent composition of claim 13 or 14 wherein the lipolytic enzyme has one, two or three hydrophobic groups covalently linked.

16. A method of preparing a dough or a baked product from the dough which
20 comprises adding to the dough a lipolytic enzyme which has at least one hydrophobic group covalently linked.

17. A dough composition comprising a lipolytic enzyme which has at least one hydrophobic group covalently linked.

Attorney Docket No.: 5885.204-US

**COMBINED DECLARATION FOR UTILITY PATENT APPLICATION
AND POWER OF ATTORNEY**

As a below named inventor, I hereby declare that:

My residence, mailing address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

Chemically modified lipolytic enzyme
(Title of the Invention)

the specification of which

☐ is attached hereto

OR

☒ was filed on (06/02/2000) as PCT International Application Number PCT/DK00/00300.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by an amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56, including for continuation-in-part applications, material information which became available between the filing date of the prior application and the national or PCT international filing date of the continuation-in-part application.

I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international applications(s) for patent or inventor's certificate or of any PCT international applications(s) which designated at least one country other than the United States of America, listed below and have also identified below any foreign application(s) for patent or inventor's certificate, or any PCT international application(s) having a filing date before that of the application(s) on which priority is claimed:

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Claimed	
PA 1999 00778	DK	06/02/1999	<input checked="" type="checkbox"/> YES	<input type="checkbox"/> NO
			<input type="checkbox"/> YES	<input type="checkbox"/> NO
			<input type="checkbox"/> YES	<input type="checkbox"/> NO
			<input type="checkbox"/> YES	<input type="checkbox"/> NO

I hereby claim the benefit under 35 U.S.C. 119(e) of any United States provisional application(s) listed below.

Application Number(s)	Filing Date
60/138,081	08/06/1999

Direct all correspondence to:

Customer Number 25908

I hereby appoint the practitioners at the above mentioned customer number as my/our attorney(s) or agent (s) to prosecute the application identified above, and to transact all business in the United States Patent and Trademark Office connected therewith.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

1-a

NAME OF SOLE OR FIRST INVENTOR	
Given Name (first and middle [if any])	Thomas Hønger
Family Name or Surname	Callisen
Inventor's Signature	<i>[Signature]</i>
Date	10-10-2001
Residence: City, State, Country	Frederiksberg, Denmark DKX
Citizenship	Danish
Mailing Address	Forchammersvej 13
City, State, Zip, Country	1920 Frederiksberg C, Denmark

2-a

NAME OF SECOND INVENTOR	
Given Name (first and middle [if any])	Shamkant Anant
Family Name or Surname	Patkar
Inventor's Signature	<i>[Signature]</i>
Date	10-10-2001
Residence: City, State, Country	Lyngby, Denmark DKX
Citizenship	Danish
Mailing Address	Christoffers Allé 91
City, State, Zip, Country	2800 Lyngby, Denmark

3-a

NAME OF THIRD INVENTOR	
Given Name (first and middle [if any])	Allan
Family Name or Surname	Svendsen
Inventor's Signature	<i>[Signature]</i>
Date	10-10-2001
Residence: City, State, Country	Hørsholm, Denmark DKX
Citizenship	Danish
Mailing Address	Overdamsvej 13
City, State, Zip, Country	2970 Hørsholm, Denmark

4-a

NAME OF FOURTH INVENTOR	
Given Name (first and middle [if any])	Jesper
Family Name or Surname	Vind
Inventor's Signature	<i>[Signature]</i>
Date	10/10/2001
Residence: City, State, Country	Værløse, Denmark DKX
Citizenship	Danish
Mailing Address	Hejrebakken 20
City, State, Zip, Country	3500 Værløse, Denmark